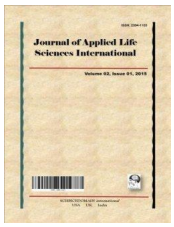

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Larvicidal Activity of *Metarhizium anisopliae* and *Annona squamosa* Leaf Extract against *Culex quinquefasciatus* and *Anopheles gambiae* Larvae

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Authors' contributions

This work was carried out in collaboration between all authors. Authors BS and EI designed the study, wrote the protocol and drafted the manuscript. Authors BS and PJM managed the experimental process. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To assess the combined efficacy of *Annona squamosa L.* leaf extract and entomopathogenic fungus *Metarhizium anisopliae* against *Culex quinquefasctiatus* and *Anopheles gambiae* larvae.
Study Design: The design of the study was experimental.
Methodology: The compatibility of *M. anisopliae* was tested on PDA treated with 100 µg/mL of *A. squamosa* extract. The mosquito larvae of *Cx. quinquefasctiatus* and *An. gambiae* were exposed to *An. squamosa* extract at 10 µg/mL, 50 µg/mL and 100 µg/mL, *M. anisopliae* at 1.7 x 10^6 conidia/ml and 1.7 x 10^7 conidia/mL, and combinations of each concentration of *A. squamosa* and *M. anisopliae.*
Results: *Metarhizium anisopliae* was compatible with *A. squamosa* and gave comparable

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germination and proliferation as it was for blank or untreated PDA. The survival of *Cx. quinquefasciatus* larvae and *An. gambiae* larvae decreased with increase in concentration of *A. squamosa* extract when tested alone or when the concentration of the extract was increased at constant concentration of *M. anisopliae.* However, an increase in concentration of *M. anisopliae* at constant concentration of *A. squamosa* extract exhibited only slight decrease in survivals as compared to the individual *M. anisopliae* treatments. On day 3, survival of *Cx. quinquefasciatus* and *An. gambiae* larvae at 50 µg/mL of *A. squamosa* crude extract were 20% and 50% respectively. However, in a combination study, 50 µg/mL of *A. squamosa* when combined with 1.7 x 10^7 conidia/mL of *M. anisopliae* caused 100% mortality of *An. gambiae* and *Cx. quinquefasciatus* within 3 days while larvicidal results for conidia alone were poor even on day 3.

Conclusion: The combination of *M. anisopliae* and *A. squamosa* showed synergistic results that could be explored further for development of mosquitoes larvicidal control agent.

Keywords: Annona squamosa; Metarhizium anisopliae; Culex quinquefasciatus; Anopheles gambiae; Larvicidal activity.

1. INTRODUCTION

Some plants species in the *Annonaceae* family have been used traditionally as insecticides [1]. For example, *A. squamosa* have been reported to have activity against mosquito larvae [2,3,4]. On the other hand entomopathogenic fungi particularly *M. anisopliae* are used as biological control agents of insects [5,6]. Various studies have been done to investigate the combination of entomopathogenic fungi with botanicals. Combine treatment of *Acalypha alnifolia* Klein ex Willd. (Euphorbiaceae) leaf extract and *M. anisopliae (Metsch.)* showed a significant efficacy on larvicidal and pupicidal activity against *Cx. quinquefasctiatus* [7]. Laboratory study reported by [8] indicated that *M. anisopliae* formulation with Neem oil significantly reduced survival of adult *Cx. quinquefasciatus* and *An. gambiae* compared to Neem oil alone. A combination of entomopathogenic fungi with plant based insecticides may provide a more suitable pest management at a reduced concentration due to combined fast effect of botanicals and slow effects of fungi on mosquito larvae. It is thus essential to determine the compatibility of botanicals with entomopathogenic fungi in order to maximize their efficacy. Since *M. anisopliae* showed substantial promise for use in integrated pest management programmes [9], the present study was done to assess compatibility of *M. anisopliae* with 20% aqueous ethanol extract of *A. squamosa* and assess individually and in combination against *Cx. quinquefasciatus* and *An. gambiae*.

2. MATERIALS AND METHODS

2.1 Treated Insects

Larvae of *Cx. quinquefasciatus* and *An. gambiae* (Culicidae) were obtained from the insectary at Muhimbili University of Health and Allied Sciences, Tanzania.

2.2 Tested Fungi

The strain of entomopathogenic fungus, *M. anisopliae* (Clavicipitaceae) used in the study was obtained from Biological Store College of Science, Swansea University in the UK.

Table 1. Combination of *M. anisopliae* and *A. squamosa* crude extract

		***M. anisopliae* (conidia/mL)**		
		0	**1.7 x 10^6**	**1.7 x 10^7**
***A. squamosa* (µg/mL)**	0	Control	0 + 1.7 x 10^6	0 + 1.7 x 10^7
	10	10 + 0	10 + 1.7 x 10^6	10 + 1.7 x 10^7
	50	50 + 0	50 + 1.7 x 10^6	50 + 1.7 x 10^7
	100	100 + 0	100 + 1.7 x 10^6	100 + 1.7 x 10^7

2.3 Tested Plant Materials

The leaves of *A. squamosa* L. (Annoneceae) were collected in September 2012 from Bagamoyo district, Cost region in Tanzania. Authentication of the plant species was done at the site with the aid of a Botanist. Air dried plant materials were ground to make fine powders which were soaked thrice with 20% aqueous ethanol. The filtrate was concentrated using rotary evaporator (*HEIDOLPH,* Germany) then completely dried using a freeze drier (EDWARDS, England).

2.4 Compatibility Test between *M. anisopliae* and *A. squamosa* Extract

The *A. squamosa* crude extract was standardized to a concentration of 100 µg/mL in PDA in a conical flask and then mixed thoroughly by gentle shaking. The medium containing extract was poured into sterilized Petri dishes to solidify. Two other portion of the PDA which saved as control contained equal volume ethanol while the second Petri dish had plane PDA. After complete solidification of the media, little fungus was introduced at the centre of the Petri dish by means of sterile micropipette base. Petri dishes were sealed with Parafilm and incubated at 25±2°C; 80±5% relative humidity for 21 days. The radial growths of *Metarhizium* species colony were measured with ruler on days 2, 6 and 10. The mean diameter of growth and proliferation was compared with that of the control using *STATA* software programme (Version 10.1). The mean differences below the probability level at P≥ 0.05 were considered statistically significant.

2.5 Methods of Treatment

2.5.1 Production of conidia harvesting and counting

Potato dextrose agar (PDA) was autoclaved at 121°C (15 psi) for 15 minutes in sterilized flask and then poured in the 10 cm diameter Petri dish. The *Metarhizium* fungal were inoculated on PDA using a sterile wire loop and then incubated at 25±2°C; 80±5% for 21 days. Conidia of *M. anisopliae* were collected over the PDA plate with an inoculation needle and rubbed gently to loosen the conidia. The vial containing 1:1 v/v water and Tween 80% was poured onto a Petri dish with conidia and then a micropipette, used to suction off the contents which were put into the vial. The conidial solution was vortexed well for few minutes to remove or to separate conidia and other mycelia. The conidial concentration of final suspension that was used in the assay was determined by direct count using haemocytometer (Neubauer Improved, *MARIENFELD,* Germany). Calculations for percentage viability were adapted as per [10].

2.5.2 Bioassay of *A. squamosa* extract, *M. anisopliae* and combined *A. squamosa* and *M. anisopliae* against *Cx. quinquefasciatus* and *An. gambiae* larvae

A stock solution of *A. squamosa* was made by dissolving 500 mg of plant extract in 10 mL of ethanol. Different dilutions with concentrations of 100, 50, and 10 µg/mL were prepared from the stock solution. Two different concentrations of *M. anisopliae* at 1.7 x 10^6 and 1.7 x 10^7 conidia/mL were made individually or in combined with *A. squamosa* extract at 10, 50 and 100 µg/mL to come out with six different combinations as shown in the matrix (above Table 1).

The larvicidal bioassay was performed according to standard WHO techniques [11]. In each bioassay, 10 larvae of the third instars were added into a beaker containing 50 mL of test concentration. Untreated mosquito larvae served as controls. All tests were carried out under controlled temperature (25±2ºC) and relative humidity (80±5%). The mortalities were recorded on day 1, 2 and 3 as percentages. Immobile larvae were considered dead [12].

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Compatibility test between *M. anisopliae* strain and *A. squamosa* extract

In vitro growth rate of *M. anisopliae* in combination with aqueous ethanol leaf extract of *A. squamosa* at concentration of 100 µg/mL using Potato dextrose agar (PDA) was studied. Growth of *M. anisopliae* exhibited similar patterns showing an increase in diameters from day 2 to 10. The growth of *M. anisopliae* was significant since by day 10 the plates were absolutely colonized with fungi and the diametersof proliferation in the plate treated with the extract and without extract showing similar pattern (Figs. 1 and 2).

The results indicate that, *M. anisopliae* in PDA containing *A. squamosa* extract did not either inhibit or enhance growth at the tested concentration. The harvested conidia, which were collected on day 21 were checked for their viability by re-inoculation into a plain PDA plate. The growth patterns of conidia harvested from treated and untreated plates were similar. Fungal proliferation increased with increase in time with day 2 diameter being 2 cm, whereas on 10^{th} day it had a colony size of 8.5 cm diameter. In general, *M. anisopliae* was compatible with *A. squamosa* and gave a well germination, proliferation and viable conidia.

3.1.2 Larvicidal activity of *A. squamosa* extracts and *M. anisopliae* individually and in combination against *Cx. quinquefasciatus* and *An. gambiae* larvae

Lavicidal activities were recorded from the first day to the third day. On the first day, 93.3% survived *Cx. quinquefasciatus* recorded at concentration of 1.7x 10^6 conidia/ mL where as it was decreased to 77% at 1.7x 10^7 conidia/mL (Fig. 3). Survivorship % of *An. gambiae* was decreased from 93.3% at a concentration of 1.7x 10^6 conidia/mL to 13.33% at 1.7x 10^7 conidia/mL on first day (Fig. 3). No mortality was observed in untreated larvae (control).

The survivorship of *Cx. quinquefasciatus* and *An. gambiae* decreased with increase in concentration of *A. squamosa* extract when tested alone or at constant concentration of *M. anisopliae* (Figs. 3, 4 and 5). For example; on first day, survivor of *Cx. quinquefasciatus* due to combination of *M. anisopliae* at 1.7 x 10^6 conidia/mL and *A. squamosa* extract at 10 µg/mL was 70% (Fig. 3), where as it was 55% when concentration of *A. squamosa* extract was increased to 100 µg/mL (Fig. 5). Similarly, 85% survived *An. gambiae* were recorded on day 1 at the same combination while it decreased to 50% mortality when the concentration of *A. squamosa* extract was 100 µg/mL (Fig. 5). A similar trend was observed for combination of *M. anisopliae* at1.7x10^7 conidia/mL and *A. squamosa* at different concentrations and for different

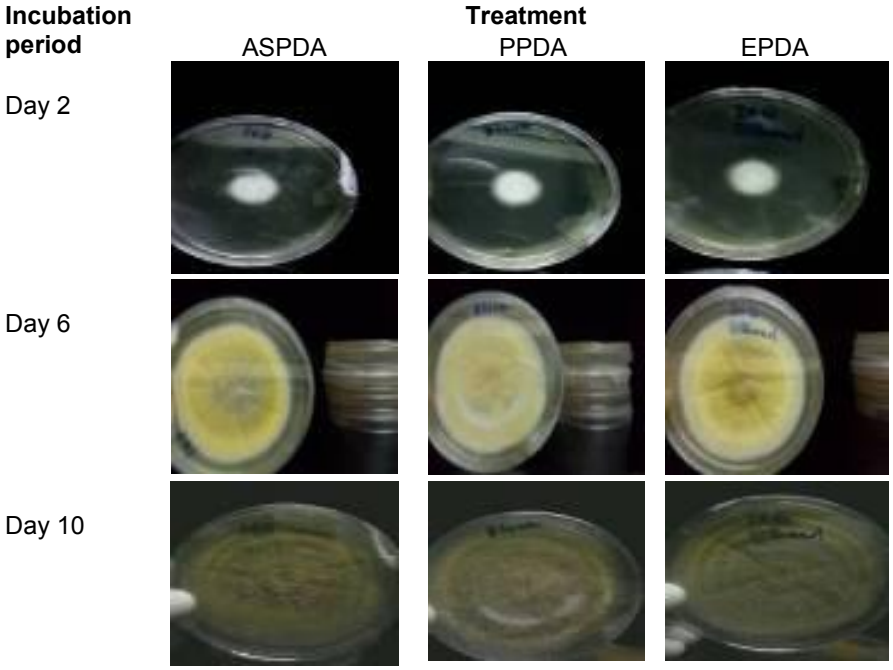

Fig. 1. Growth rate of different strain of *M. anisopliae* in Potato Dextrose Agar (PDA) with and without *A. squamosa* leaf extract on day 2, 6 and 10

ASPDA= M. anisopliae sub cultured on Potato Dextrose Agar contaminated with 100 µg/mL of A. squamosa extract. PPDA= M. anisopliae sub cultured on plain Potato Dextrose Agar. EPDA= M. anisopliae sub cultured on Potato Dextrose Agar contaminated with ethanol

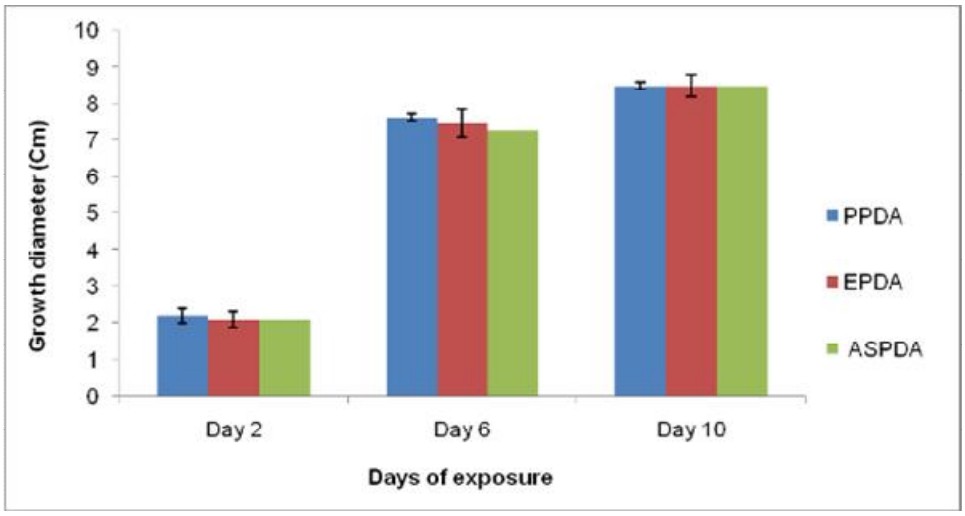

Fig. 2. Effect of *A. squamosa* leaf extract on growth rate of different strain of *M. anisopliae* on day 2, day 6 and day 10

PPDA= M. anisopliae sub cultured on Plain Potato Dextrose Agar. EPDA= M. anisopliae sub cultured on Potato Dextrose Agar contaminated with ethanol. ASPDA= M. anisopliae sub cultured on Potato Dextrose Agar contaminated 100 µg/mL of A. squamosa extract

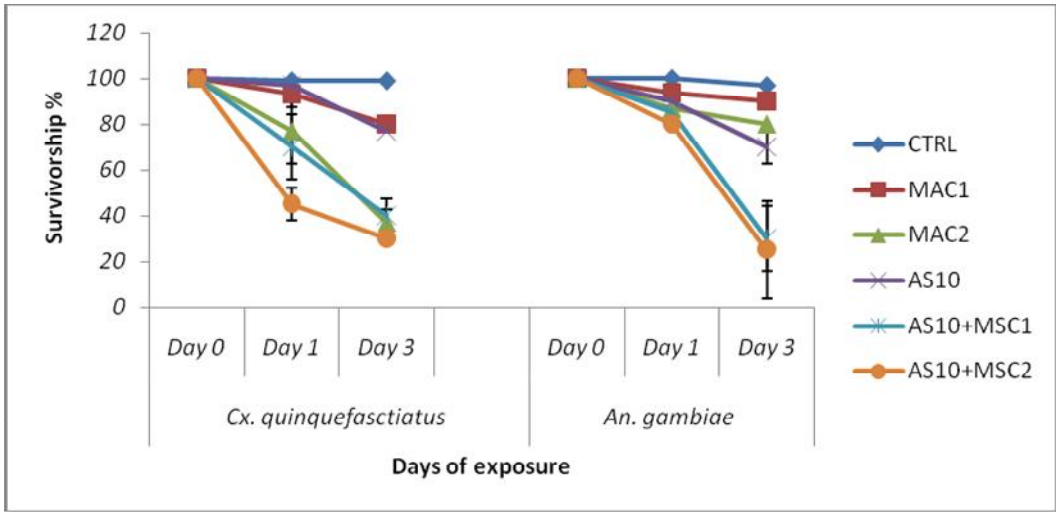

Fig. 3. Survivorship % of *Cx. quinquefasciatus* and *An. gambiae* after exposure to different concentrations of *M. anisopliae* at 10 µg/mL of *A. squamosa* extract

CTRL= Untreated mosquito larvae (Control). MAC1= Mosquito larvae exposed to 1.7 x 10^6 conidia/mL of M. anisopliae. MAC2= Mosquito larvae exposed to 1.7 x 10^7 conidia/mL of M. anisopliae. AS10= Mosquito larvae exposed to 10 µg/mL of A. squamosa. AS10+MAC1= Mosquito larvae exposed to 1.7 x 10^6 conidia/mL of M. anisopliae and 10 µg/mL A. squamosa. AS10+MAC2 = Mosquito larvae exposed to 1.7 x 10^7 conidia/mL of M. anisopliae and 10 µg/mL A. squamosa

exposure time. On the other hand, increase in concentration of *M. anisopliae* at constant concentration of *A. squamosa* extract exhibit only slight impact on survived larvae. For example in Fig. 3; Survived larvae on the 3rd day of the combination of *A. squamosa* at 10 µg/mL and 1.7 x 10^6 conidia/mL of *M. anisopliae* was 40% against *Cx. quinquefasciatus* and 30% against *An. gambiae*. An increase in concentration to1.7 x 10^7 conidia/mL during the same exposure time caused a decrease in survival to only 30% of *Cx. quinquefasciatus* and 25% survived for *An. gambiae* a reduction that was not significant different ($p>0.05$) among the combination treatments of the same *A. squamosa* extract.

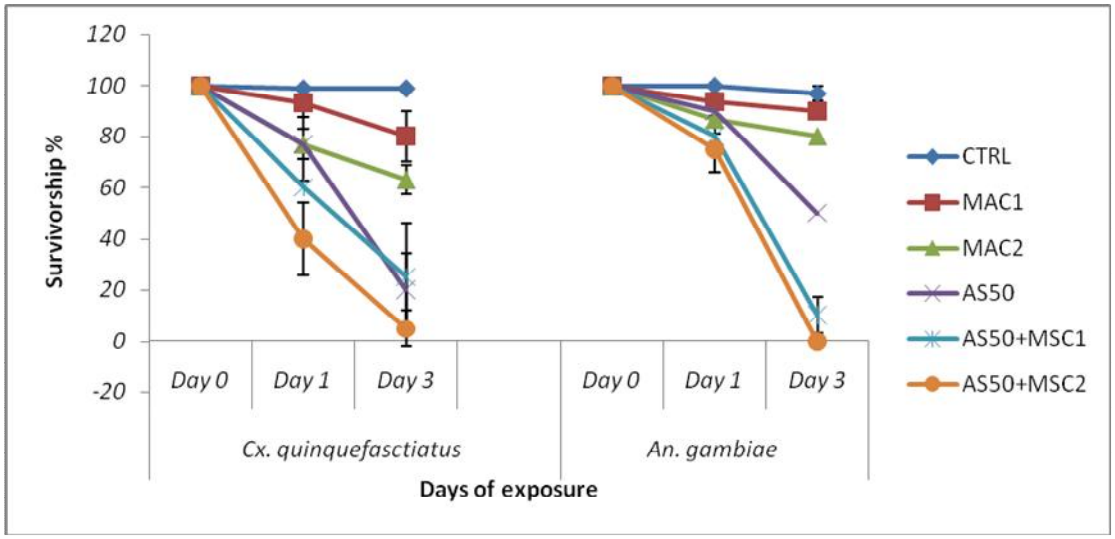

Fig. 4. Survivorship % of *Cx. quinquefasciatus* and *An. gambiae* after exposure to different concentrations of *M. anisopliae* at 50 µg/mL of *A. squamosa* extract

CTRL=Untreated mosquito larvae (control). MAC1= Mosquito larvae exposed to 1.7 x 10^6 conidia/mL of M. anisopliae. MAC2= Mosquito larvae exposed to 1.7 x 10^7 conidia/mL of M. anisopliae. AS50= Mosquito larvae exposed to 50 µg/mL of A. squamosa. AS50+MAC1= Mosquito larvae exposed to 1.7 x 10^6 conidia/mL of M. anisopliae and 50 µg/mL A. squamosa. AS50+MAC2 = Mosquito larvae exposed to 1.7 x 10^7 conidia/mL of M. anisopliae and 50 µg/mL A. squamosa

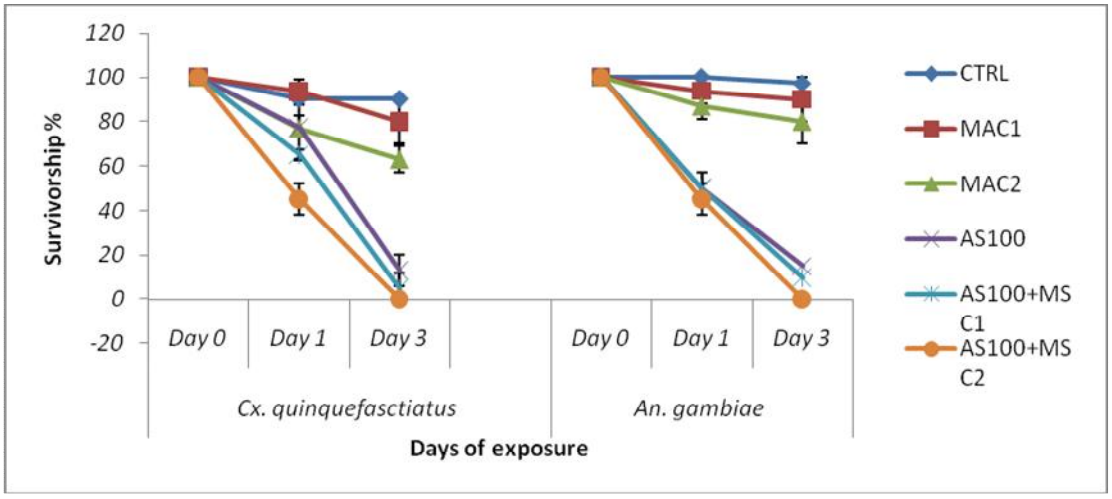

Fig. 5. Survivorship % of *Cx. quinquefasciatus* and *An. gambiae* after exposure to different concentrations of *M. anisopliae* at 100 µg/mL of *A. squamosa* extract

CTRL= Untreated mosquito larvae (control). MAC1= Mosquito larvae exposed to 1.7 x 10^6 conidia/mL of M. anisopliae. MAC2= Mosquito larvae exposed to 1.7 x 10^7 conidia/mL of M. anisopliae. AS100= Mosquito larvae exposed to 100 µg/mL of A. squamosa. AS100+MAC1= Mosquito larvae exposed to 1.7 x 10^6 conidia/mL of M. anisopliae and 100 µg/mL A. squamosa. AS100+MAC2 = Mosquito larvae exposed to 1.7 x 10^7 conidia/mL of M. anisopliae and 100 µg/mL A. squamosa

3.2 Discussion

In the presence study, the numbers of live mosquito larvae were significantly ($p<0.05$) reduced as a result of exposure to the combination treatment as compared to exposure to the individual treatment. Previous studies showed that combination of *M. anisopliae* and plants had a potential synergy activity against mosquito larvae [13]. The synergy may be due to

combined effect of the fast and slow acting nature of some botanicals and fungi. Moreover, *M. anisopliae may* require several biological and mechanical processes that are why mortalities could only be seen after day 3. Mechanical action of *M. anisopliae* may cause larvae mortality within 2 to 7 days due to conidia germination and hyphae penetration through haemocoel [14]. Furthermore, conidia behaviors in aqueous solution tend to form clusters at the bottom of the beaker due to their lypophilic nature [15]. This could have caused poor contact with larvae as they spend more time at the surface of water for respiration. The examination of compatibility between *A. squamosa* and *M. anisopliae* was important component for production of viable mosquito larval control agents. Conidia germination and proliferation is the most significant characteristic in initiating biological control of *M. anisopliae*, also the primary step for the initiation of infection procedure in insects [16,17]. According to the results of this study, *M. anisopliae* was compatible with *A. squamosa* to warrant viable control agent for *Cx*. *quinquefasciatus* and *An. gambiae* larvae.

5. CONCLUSION

Combined *M. anisopliae* and *A. squamosa* leaf crude extract showed higher lavicidal activity against *Cx. quinquefasciatus* and *An. gambiae* as compared to *A. squamosa* leaf extract and *M. anisopliae* alone, hence promising mosquito control agent for development.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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